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<b>(54) Title:</b> PARATHION HYDROLASE ANALOGS AND METHODS FOR PRODUCTION AND PURIFICATION			
<b>(57) Abstract:</b> The present invention provides a novel gene encoding DNA sequence for the expression of parathion hydrolase and an analog thereof, and methods for preparing such compounds. In a particularly preferred embodiment, the present invention relates to a parathion hydrolase gene encoding sequence in which twenty-eight amino acids are deleted from the N-terminal amino acid sequence of parathion hydrolase. The novel gene encoding parathion hydrolase encoding sequence is useful for production of the mature or processed parathion hydrolase and a methionine (-1) analog thereof.			

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- 1 -

PARATHION HYDROLASE ANALOGS  
AND METHODS FOR PRODUCTION AND PURIFICATION

5        This is a continuation-in-part of  
Ser. No. 237,255 filed August 26, 1988 hereby  
incorporated by reference.

10      The present invention provides a novel gene  
encoding DNA sequence for the expression of parathion  
hydrolase and an analog thereof, and a method for  
preparing such compounds. Other synonyms for parathion  
hydrolase include organophosphorus acid anhydrolase  
parathion aryl esterase, and phosphotriesterase. In a  
particularly preferred embodiment, the present invention  
15      relates to a parathion hydrolase gene encoding sequence  
in which twenty-eight amino acids are deleted from the  
N-terminal amino acid sequence of parathion hydrolase.  
The novel gene encoding parathion hydrolase encoding  
sequence is useful for production of the mature or  
20      processed parathion hydrolase and a methionine (-1)  
analog thereof.

BACKGROUND

25      Parathion (0,0-diethyl-O-p-nitrophenyl  
phosphorothioate) was discovered in 1944 by Schrader  
(Eto, M., 1974 organophosphorus pesticides: organic and  
biological chemistry, CRC Press Inc., Cleveland, Ohio).  
It is an organophosphate pesticide that is extremely  
30      toxic to higher organisms because parathion (PAR) is  
converted by the cytochrome P-450 monooxygenase system  
to paraoxon which is a potent acetylcholinesterase  
inhibitor (Taylor, The pharmacological basis of  
therapeutics, 6th ed. pp. 100-119 (1980)).

- 2 -

Organophosphate pesticides applied to agricultural environments at recommended rates are not extremely persistent. However, when higher concentrations of PAR are applied to soil samples, 5 persistence increases, and PAR can be detected in some soils after 16 years (Wolfe et al., Bull. Environm. Contam. Toxicol. 10:1-9 (1973)). It has been estimated that the remaining pesticide residue in pesticide containers is approximately  $4.5 \times 10^5$  kilograms per year 10 (Munnecke, Process Biochem. 13:16-19 (1978)). The major source of pesticide discharges occurs at pesticide production and formulation plants, which release more than  $4 \times 10^5$  kilograms of pesticides each year 15 (Munnecke, supra (1978)). Therefore, hazards exist with respect to undesirable discharges such as pesticide containers, production plants, or accidental spills 20 (Munnecke, Microbial degradation of xenobiotics and recalcitrant compounds, Academic Press Inc., London, pp. 251-270 (1981)). In these instances, clean-up procedures are required. Enzymes which can decrease the 25 toxicity of the pesticide molecule by a simple hydrolysis reaction could possibly be used for the detoxification of residual pesticides at these industrial point sources.

25

1. Microbial Degradation of Organophosphates and Possible Industrial Applications

Over millions of years, microorganisms have 30 evolved the ability to utilize many compounds as sole sources of carbon and energy (Gibson et al., Microbial degradation of organic compounds, Marcel Dekker, Inc. New York (1984)). The importance of the studies of 35 microbial metabolism is exemplified by the virtue of microorganisms as the major means by which many chemical pollutants are eliminated from a variety of ecosystems.

- 3 -

The vast majority of the 500 or so active pesticidal chemicals can be biologically degraded by fungal or bacterial microorganisms. General reviews discussing microbial metabolism of pesticides have been 5 edited by Bollag, *Advan. in App. Micro.* 18:75-130 (1974), and Laveglia and Dahm, *Annu. Rev. Entomol.* 22:483-513 (1977).

Munnecke and Hsieh, *Appl. Environ. Microbiol.* 31:63-69 (1976) have studied the degradation 10 of parathion by a mixed microbial culture and found the major metabolites to be *p*-nitrophenol and diethylthiophosphoric acid (Figure 1). Hydrolysis of parathion reduces its toxicity by nearly 120-fold and releases water-soluble metabolites that are more 15 available to further microbial degradation. *p*-nitrophenol can be further utilized as a source of carbon and energy by other microorganisms (Munnecke and Hsieh, *supra*; Simpson, *Biochem. J.* 55:xxiv (1953); and Spain et al., *Biochem. Biophys. Res.* 20 *Commun.* 88:634-641 (1979)). Another study has shown that diethylthiophosphoric acid is utilized as the sole phosphorus source by some soil microorganisms (Cook et al., *Appl. Environ. Microbiol.* 36:668-672 (1978)). A cell-free enzyme preparation obtained by 25 Munnecke from a mixed bacterial culture hydrolyzed the organophosphate insecticides ethyl parathion, methyl parathion, paraoxon, diazinon, dursban, EPN, cyanophos, fenitrothion, and triazophos at rates significantly higher than chemical hydrolysis with sodium hydroxide 30 (Munnecke, *Appl. Environ. Microbiol.* 32:7-13 (1976)). The enzyme activity for pesticide hydrolysis was stable at temperatures up to 45-50°C and had a temperature and pH optimum for expression of enzymatic activity of 35°C and pH 8.5-9.0, respectively (Munnecke, *supra* (1976)).

- 4 -

Parathion is enzymatically hydrolyzed to diethylthiophosphoric acid (I) and p-nitrophenol (II) by parathion hydrolase for P. diminuta MG (pCMS1) (Figure 1).

5 The enzymatic removal of PAR from pesticide containers has been demonstrated (Munnecke, Agric. Food Chem. 28:105-111 (1980)). It was observed that within 16 hours, 94% of the residual PAR present in the drums as a 48% emulsifiable concentrate was hydrolyzed after 10 parathion hydrolase was added.

The feasibility of using enzyme preparations to clean up soil spills has been demonstrated in both laboratory and field conditions. Work by Barik and Munnecke, Bull. Environm. Contam. Toxicol. 29:235-239 15 (1982) has shown that parathion hydrolase can hydrolyze diazinon in soil. More than 98% of 1% diazinon in the soil was removed within 24 hours when sufficient amounts of buffer and enzyme were added to the contaminated soil. This concentration of pesticide would normally 20 remain in untreated soil for many years (Wolfe et al., supra). In greenhouse studies, soil samples were treated with different concentrations of diazinon (Honeycutt et al., ACS Symposium Series, No. 259, Treatment & Disposal of Pesticide Wastes, pp. 343-352 25 (1984)). Parathion hydrolase was added to the soil samples to determine the efficacy of the enzyme to rapidly degrade diazinon during a spill situation. The half-life of diazinon in the 500 ppm treatment without enzyme present was 9.4 days, while the half-life of 30 diazinon in the 500 ppm treatment with enzyme present was one hour. These studies indicate that parathion hydrolase can be used effectively to rapidly reduce large concentrations of diazinon in soil.

Investigations using a mixed microbial culture 35 that converts PAR to PNP led to the isolation of Pseudomonas diminuta strain MG which contains high

- 5 -

levels of parathion hydrolase activity (Serdar et al., Appl. Environ. Microbiol. 44:246-249 (1982)). In all cases, parathion hydrolase activity was produced in the absence of added PAR. On the basis of these results, it 5 appears that parathion hydrolase was produced constitutively by this organism. P. diminuta MG and a Flavobacterium sp. (ATCC 27551), isolated by Sethunathan and Yoshida, Can. J. Microbiol. 19:873-875 (1973), are the most extensively studied organisms that degrade 10 organophosphates.

## 2. Plasmids Involved in Organophosphate Degradation

Plasmids are extrachromosomal genetic elements 15 that code primarily for nonessential functions yet enable certain organisms to survive in unique ecological niches. Many unusual metabolic activities of Pseudomonas species are plasmid-encoded functions (Haas, Experientia, 39:1199-1213 (1983)).

To determine whether parathion hydrolase 20 activity was controlled by a plasmid, attempts have been made to correlate loss of PAR hydrolase activity with plasmid removal. Expression of enzymatic activity was lost at a frequency of approximately 12% after treatment 25 with mitomycin C (Serdar, supra (1982)). Hydrolase-negative derivatives were lacking a plasmid present in the wild-type organism. These cured colonies differed phenotypically from the parent strain only in inability 30 to hydrolyze PAR. These results suggested that PAR hydrolase activity may be mediated by plasmid DNA, although induction of deletion mutations could not have been excluded.

This plasmid designated as pCMS1 was 35 determined to be 66 kilobase (kb) in size by using electron microscopy (Serdar, supra (1982)). Recent studies using restriction mapping have confirmed the

- 6 -

molecular size estimate (Mulbry et al., *Appl. Environ. Microbiol.* 51:926-930 (1986)). This is the first example of plasmid involvement in the degradation of an organophosphorus compound.

5 More recently it was shown that the Flavobacterium sp. ATCC 27551 contains a 39 kb plasmid, pPDL2, that is involved in the degradation of organophosphates (Mulbry et al., supra (1986); and Mulbry et al., *Plasmid* 18:173-177 (1987)). The gene  
10 encoding parathion hydrolase (termed opd, organophosphate-degrading gene) from pCMS1 was shown to be homologous to that located in pPDL2, as determined by DNA-DNA hybridization and restriction mapping. Further DNA hybridization studies have revealed that these  
15 plasmids share homology only within an approximately 5.1 kb region of DNA (Mulbry et al., supra (1987)).

### 3. Construction of Recombinant Plasmids Containing the Parathion Hydrolase Gene

20 Two cloning strategies were employed (Serdar and Gibson, *Bio/Technology* 3:567-571(1985)). One method was the use of different restriction endonuclease sites of pBR322 (Bolivar et al., *Gene*. 2:95-113(1977)). for  
25 'shot-gun' cloning with subsequent studies for parathion hydrolase expression in E. coli. The other procedure utilized the EcoRI site of the broad host range vector, pKT230 (Bagdasarian et al., *Gene*. 16:237-247(1981)); recombinant plasmids generated using pKT230 can be  
30 mobilized into Pseudomonas strains using a helper plasmid, pRK2013 (Figurski et al., *Proc. Natl. Acad. Sci. USA*. 76:1648-1652(1979)).

Following the cloning experiment using pKT230 as the vector, two transformants that hydrolyzed PAR  
35 were isolated, and each was shown to contain a hybrid plasmid with an identical 6.0 kilobase EcoRI insert

- 7 -

(Serdar, supra(1985)). This plasmid was designated as pCMS29 as shown in Figure 2.

Figure 2 represents the physical maps of the recombinant plasmids carrying the parathion hydrolase gene of P. diminuta MG (pCMS1). The thick lines represent the adjacent portions of vector DNA; the thin lines represent the inserted pCMS1 DNA fragments. Restriction endonucleases used were: B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SalI; T, TagI; X, XbaI.

Alternative cloning procedures utilized the BamHI, HindIII and SalI restriction sites of the vector pBR322. DNA fragments produced from pCMS1 by these restriction enzymes were ligated into similarly treated pBR322 and were used to transform E. coli HB101. A BamHI-generated clone which hydrolyzed PAR was shown to contain a hybrid plasmid designated as pCMS5 (Figure 2). Analysis by restriction endonuclease digestion revealed that this plasmid contained a 1.5 kb insert. In contrast, parathion hydrolase activity was not detected in any of the transformants containing hybrid plasmids constructed with SalI.

In order to increase the synthesis of parathion hydrolase in E. coli and to construct a 1.5 kb fragment suitable for inserting into the EcoRI site of the plasmid pKT230, the 1.5 kb BamHI fragment located in pCMS29 was subcloned into the high expression vector pUC7 (Vieira et al., Gene. 19:259-268(1982)). This resulting strain contained a plasmid, designated as pCMS40, with a 1.5 kb insert which was flanked by the EcoRI sites of pUC7. Restriction maps of the 6.0 kb EcoRI fragment in pCMS29, the 1.5 kb BamHI fragment in pCMS5, and the subcloned 1.5 kb BamHI fragment in pCMS40 are shown in Figure 2.

Plasmids suitable for studying the expression of the parathion hydrolase gene in Pseudomonas strains were constructed by EcoRI cleavage of pCMS40 and subsequent ligation into EcoRI cleaved pKT230.

- 8 -

Table I. Parathion hydrolase activity\* (Prior Art)

	Strain	Activity
5	<u>P. diminuta</u> MG	ND
	<u>P. diminuta</u> MG (pCMS1)	2.1
	<u>P. diminuta</u> MG (pCMS29)	4.8
	<u>P. diminuta</u> MG (pCMS55)	9.2
10	<u>Pseudomonas</u> strain 24	ND
	<u>Pseudomonas</u> strain 24 (pCMS29)	0.03
	<u>Pseudomonas</u> strain 24 (pCMS55)	0.07
 <u>E. coli</u> strains		
15	HB101 (pBR322)	ND
	BHB2600 (pKT230)	ND
	JM105 (pUC7)	0.002
	BHB2600 (pCMS29)	0.002
	HB101 (pCMS5)	0.03
	JM105 (pCMS40)	0.19
	BHB2600 (pCMS55)	0.004
20	BHB2600 (pCMS60)	ND

\*Activity has been converted from nanomoles to micromoles ( $\mu$ mol) of parathion hydrolyzed per minute per milligram of protein. ND, not detected.

25                   E. coli BHB2600 cells transformed with plasmids designated pCMS55 and pCMS58 showed enzymatic activity, and each plasmid contained the 1.5 kb BamHI insert. The highest parathion hydrolase activity was 30 associated with pCMS55 which contained two 1.5 kb inserts in the same orientation relative to the vector as shown in Figure 3. Further screening led to the isolation of an additional strain that contained a plasmid (pCMS60) with the same 1.5 kb insert in the 35 opposite orientation. However, this strain showed no parathion hydrolase activity. Figure 3 represents the

- 9 -

physical maps of pKT230 derivatives containing the parathion hydrolase gene in different orientations. The heavy lines correspond to the pKT230 DNA; the thin lines show the inserted pCMS1 DNA. Restriction endonucleases used were: B, BamHI; E, EcoRI, P, PstI; S, SalI; X, XhoI.

Of particular interest to the background of the present invention is McDaniel et al., J. Bacteriol., 170:2306, 1988, which published a DNA sequence of a 10 1.32 kb PstI fragment from P. diminuta MG plasmid, pCMS1. McDaniel et al. observed the total number of base pairs (bp) to be 1322. According to their analysis the stop codon (TGA) was located at the unique DdeI site within the parathion hydrolase gene at 1038 bp. The open 15 reading frame was determined to be 975 bp. McDaniel et al. constructed several subclones: two clones extended the 3'-end to approximately 675 bp and 840 bp. These strains, containing either clone, had no enzymatic activity. Another construct was deleted at the 5'-end 20 SalI site (353 bp) which also did not contain activity.

McDaniel et al. concluded that the parathion hydrolase enzyme was not soluble and was associated with the particulate fraction of lysed P. diminuta cells. They suggested that the enzyme was released from the 25 particulate fraction upon treatment with detergents such as Triton X-100 or Tween 20. From their evaluation of elution fractions from Sephadex G-200 column chromatography, they concluded that the molecular weight of the enzyme was 60-65 kilodaltons (kd). From their 30 molecular weight determinations, McDaniel et al. suggested that the enzyme exists in its active form as a dimer. The McDaniel et al. article provides no evidence that the enzyme produced is processed parathion hydrolase.

To date, a correct gene encoding sequence for 35 unprocessed parathion hydrolase has not been described. In particular, no gene encoding sequence for processed parathion hydrolase has been described or suggested.

- 10 -

#### SUMMARY OF THE INVENTION

It should be noted that, as employed herein, the term parathion hydrolase refers to compounds derived 5 from a wild-type strain or having host cell systems having the amino acid sequence as shown in Figure 6. The gene encoding sequence for parathion hydrolase, as shown in Figure 6, starts at position 67 bp and provides an unprocessed parathion hydrolase product having a 10 molecular weight of approximately 39 kd as indicated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The term processed parathion hydrolase refers to a compound derived from a host cell system having a gene encoding sequence as shown in Figure 6. The gene encoding 15 sequence as shown in Figure 6 starts at position 154 bp and provides a processed parathion hydrolase product having a molecular weight of approximately 36 kd as indicated by SDS-PAGE.

The present invention provides higher purity of 20 processed parathion hydrolase that is obtained from crude or partially purified extracts. This is the first demonstration of obtaining a high level of parathion hydrolase activity in an alternative host system such as E. coli.

25 The present invention provides processed parathion hydrolase in soluble form in commercial quantities that is stable.

The present invention provides a gene encoding sequence for expression or production of processed 30 parathion hydrolase and a methionine (-1) analog thereof. The present invention also provides an industrially feasible recombinant process for preparing high levels of active and stable processed parathion hydrolase. The gene encoding sequence and analogs 35 thereof, according to the present invention, are generally characterized by the deletion of twenty-nine

- 11 -

amino acids from the N-terminal amino acid sequence of parathion hydrolase.

The processed parathion hydrolase product of the present invention has an amino acid sequence wherein 5 the N-terminal amino acid sequence of the processed parathion hydrolase starts with serine at the 30th position. Alternatively, the processed parathion hydrolase is a methionine (-1) analog.

10 A nucleic acid according to the present invention has codons encoding a polypeptide analog as described above.

15 A system for the production of processed parathion hydrolase according to the present invention comprises a host cell in turn comprising nucleic acid encoding a processed parathion hydrolase or a methionine 20 (-1) analog thereof. In such a cell, the nucleic acid encoding the processed parathion hydrolase or analog may be chromosomal or extrachromosomal. The host cell is preferably selected from the group consisting of 25 E. coli, Bacillus and Streptomyces.

An object of the present invention is to provide a process for producing high activity levels of processed parathion hydrolase according to the system described above which further includes the addition of 25 metals such as Cobalt (Co) and Zinc (Zn) in sufficient amounts to increase the activity of enzyme greater than 20 fold. In a preferred embodiment, amounts of > 0.1 mM of cobalt and/or zinc have significantly increased the activity of enzyme.

30 Yet a further object of the present invention is to provide a method of purification of soluble and processed parathion hydrolase in the absence of detergents such as Triton X-100 and Tween 20.

35 Further aspects and advantages of the present invention will become apparent upon consideration of the following detailed description of presently preferred embodiments thereof.

- 12 -

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the hydrolysis of parathion. (Prior Art)

5

Figure 2 shows the physical maps of recombinant plasmids carrying the parathion hydrolase gene of P. diminuta MG (pCMS1). (Prior Art)

10

Figure 3 shows the physical maps of KT230 derivatives containing the parathion hydrolase in different orientations. (Prior Art).

15 Figure 4 shows a linear map of the recombinant plasmids of the present invention.

Figure 5 shows the sequencing strategy of the 1.34 Kb PstI fragment.

20

Figure 6 shows the nucleotide sequence of the 1.34 Kb PstI fragment.

DETAILED DESCRIPTION

25 The methods and materials which provide an illustration of the invention and which comprise the presently preferred embodiment relate to a gene encoding sequence which can be employed in the production of processed parathion hydrolase.

30

The invention is now illustrated by the following Examples, with reference to the accompanying drawings. The examples do not include detailed descriptions for conventional methods employed in the isolation of DNA, the cleavage of DNA with restriction enzymes, the construction of vectors, the insertion of DNA gene segments encoding polypeptides of interest into

- 13 -

such vectors (e.g. plasmids), or the introduction of the resulting recombinant plasmids into microorganism host cells. Such methods are well known to those skilled in the art of genetic engineering and are described in numerous publications including the following:

5 Maniatis et al., Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory (1982); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing Co. (1986); Current Protocols in Molecular Biology, edited by Ausubel et al., Greene Publishing Associates and Wiley Interscience (1987).

In order to determine the correct gene encoding sequence for unprocessed parathion hydrolase, a series of plasmids were constructed to determine the correct location of the parathion hydrolase gene within the pCMS1 DNA. It should be noted that in contrast to the constructs of McDaniel et al., supra, that did not yield enzyme activity, the following constructs were constructed to specifically determine the proper identification of the coding region. Plasmids pCMS70, pCMS71, pCMS72, pCMS73, and pCMS74 consist of pCMS1 DNA, extending from the PstI site to BamHI, XbaI, BclI, DdeI and PstI sites, respectively, as shown in Figure 4. The linear map of the recombinant plasmids constructed in this study are shown in Figure 4. Only relevant restriction sites used for subcloning are indicated. The thick lines represent the adjacent portions of vector DNA; the thin lines represent the inserted DNA. The arrow indicates the direction of transcription and the extent of the parathion hydrolase gene as determined by DNA sequence analysis. It includes a functional mapping of the parathion hydrolase gene and the phenotype of the strains containing these plasmids as indicated: (+) crude cell extracts contain parathion hydrolase activity, (-) crude cell extracts do not contain parathion hydrolase activity. These fragments were

- 14 -

constructed to identify the coding region and to confirm the DNA sequence results. None of the strains containing plasmids where the 3'-end was terminated at the 818 bp XbaI site (pCMS71), 913bp BclI site (pCMS72), 1054 bp 5 DdeI site (pCMS73) and at 1089 bp (extended from the DdeI site using a linker, pCMS76) showed any enzyme activity.

The plasmids constructed to determine the boundaries of the coding region and to confirm the DNA sequence data are as follows:

10

Construction of pAC1 plasmid vector

The starting material for the construction of the pAC1 plasmid was the pCFM526 plasmid. A detailed 15 description of the construction of expression vectors, including pCFM526, is described in U.S. Patent 4,710,473, which is hereby incorporated by reference in its entirety. The pCFM526 plasmid contains a heat inducible promoter, a restriction site bank (cloning 20 cluster), plasmid origin of replication, a transcription terminator, genes regulating plasmid copy number, gene conferring ampicillin resistance, and a synthetic ribosome binding site immediately preceding the cloning cluster. The pAC1 plasmid was derived from pCFM526 by 25 substituting the DNA sequence between the unique AatII and ClaI restriction sites with the following oligonucleotide.

AatII

30 CAGATCCATAAATTATCTCTGGCGGTGTTGACATAAATACCACTGGCGGTGATAA-  
TGCAGTCTAGGTATTTAATAGAGACCGCCACAACTGTATTTATGGTGACCGCCACTATT-

ClaI

-TGAGCACAT  
35 -ACTCGTGTAGC

- 15 -

This was performed by digesting pCFM526 with AatII and ClaI and isolating from agarose gel a 4.0 kb fragment which was ligated to the above oligonucleotide.

5

PCMS70

A 1200 bp PstI to BamHI fragment containing the parathion hydrolase gene was released from the plasmid pCMS40 and was cloned into Amgen vector pAC1. 10 The cloning was done by digesting pCMS40 with PstI and treating with Klenow to blunt the PstI sticky ends. The DNA was digested with BamHI following phenol extraction. The pAC1 vector was digested with HpaI, phenol extracted, and then digested with BamHI. Both 15 insert and vector DNAs were isolated from agarose gel and ligated. The resulting plasmid was designated as pCMS70.

PCMS71

20

The pCMS70 plasmid was digested with XbaI. Since an XbaI site is located in the pAC1 vector 10 bp upstream from the PstI/HpaI junction, a 830 bp fragment was released from the plasmid. Plasmid pAC1 DNA was 25 digested with XbaI and treated with phosphotase. Both the vector and 830 bp insert DNAs were isolated from agarose gel and ligated. The resulting correctly oriented plasmid was designated as pCMS71.

30

PCMS72

Plasmid pCMS70 DNA was transformed into Dam- strain of E. coli GM119 (CGSC Strain #6475) to cleave the BclI site. Plasmid DNA obtained from this strain 35 was then digested with BclI and BamHI in order to eliminate a 260 bp fragment from the 3' end of the

- 16 -

plasmid. The remaining plasmid DNA was isolated from agarose gel and religated to itself. This plasmid was designated as pCMS72.

5 pCMS73

The pCMS70 DNA was digested with ClaI and BamHI releasing a 1200 bp insert which was isolated from agarose gel. This DNA was then digested with DdeI 10 releasing a 115 bp of DNA from the 3' end of the gene. The digest mixture was treated with Klenow and digested with EcoRI to eliminate a 50 bp fragment from the 5' end of the DNA insert. This treatment gave rise to a 1050 bp EcoRI/ DdeI blunt ended DNA fragment. Plasmid pAC1 15 vector DNA was digested with HpaI and EcoRI. Both vector and insert DNAs were isolated from agarose gel and ligated. The resulting plasmid was designated as pCMS73.

20 pCMS74

This plasmid was constructed by isolating a 1.34 Kb PstI fragment from pCMS29 (Serdar, supra 1985). Following the Klenow treatment to form blunt 25 ends, this fragment was inserted into HpaI digested pAC1 by ligation. Correct orientation was confirmed by restriction mapping of clones having parathion hydrolase activity.

30 pCMS76

This construct was made by first digesting the pCMS75 (described below) DNA with ClaI/BamHI double digest and isolating the 1100 bp insert from agarose. 35 This insert DNA was then digested with DdeI yielding a 960 bp ClaI/DdeI fragment which was isolated from

- 17 -

agarose gel. Plasmid pAC1 was digested with Clal/BamHI double digest and isolated from agarose gel. A synthetic oligomer was designed with 5' DdeI and 3' BamHI sticky ends. The internal codons were kept the same as the original sequence, and a TAG termination codon was placed after the valine codon (pos. 1089). The resulting plasmid was designated as pCMS76.

DNA sequence of parathion hydrolase gene

10

To reveal the exact coding region, the 1.34 kb PstI fragment in plasmid pCMS74 was sequenced as shown in Figure 5. The relevant restriction map, sequencing strategy, and subclones constructed for sequencing is indicated with restriction sites used for sequencing or subcloning. Arrows represent the direction and extent of the sequences obtained by using fragments produced by restriction enzyme digestion at the sites indicated or sequences obtained using synthetic oligonucleotides as primers.

The sequence disclosed a single open reading frame with an initiation codon located near the PstI site. Although, McDaniel et al., supra, believed the total number of base pairs to be 1322, the PstI fragment is 1341 base pairs as shown in Figure 6. The nucleotide sequence of the 1.34 kilobase PstI fragment of pCMS1 containing the parathion hydrolase gene is indicated. The sequence shown is 5'->3'. The deduced amino acid sequence of the parathion hydrolase gene product is given below the DNA sequence. The sequences were determined in both directions by the Sanger method (Sanger, F. et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977); Heidecker, G., et al., Gene 10:69-73 (1980)). Relevant restriction sites are indicated. The experimentally determined N-terminal amino acid sequences are also double underlined.

- 18 -

The coding region (open reading frame) is 1095 bp, starting at an ATG site located 67 bp from the PstI site. The stop codon (TGA) was located at 1162 bp which is 103 bp downstream of the unique DdeI site (1054 bp) 5 within the parathion hydrolase gene. This site is 8 bp upstream of the BamHI site located at 1173 bp.

A construct of the parathion hydrolase gene based on the results of the N-terminal amino acid sequence was generated. Purified parathion hydrolase 10 (see example 2) was used for N-terminal amino acid analysis. Protein sequence analyses were performed by automated Edman degradation in an ABI 470A gas-phase microsequenator (Hewick, R.M. et al., J. Biol. Chem. 256: 7990-7997 (1981); Hunkapiller, M.W. et al., 15 Meth. Enzymol. 91:399-413 (1985) The N-terminal amino acid sequence was identified as:

Ser-Ile-Gly-Thr-Gly-Asp-Arg-Ile-Asn-Thr-Val-Arg-Gly-Pro-Ile

20 Preferred Embodiments

Preferred embodiments of novel plasmids of the invention include plasmid pCMS75 which was deposited with the American Type Culture Collection, on 25 August 25, 1988 and designated A.T.C.C. No. 67778; and pCMS77 which was deposited with the American Type Culture Collection, on August 25, 1988 and designated A.T.C.C. NO. 67779. The insert in the expression vector consisting of the BamHI site at the 3'-end contained 30 parathion hydrolase activity.

pCMS75

Plasmid pAC1 was digested with XbaI/BamHI and 35 the fragment was isolated from agarose gel. The ClaI/BamHI fragment that was released by ClaI/BamHI

- 19 -

double digest of the pCMS70 plasmid was isolated from agarose gel and subsequently digested with AvaII. This resulted in a 990 bp AvaII/BamHI DNA fragment which was isolated from agarose gel. A synthetic linker was 5 designed with 5' XbaI and 3' AvaII sticky ends. The linker was designed to allow proper spacing between the XbaI site of the pAC1 expression vector and the ATG start site. The ATG codon was followed by a serine codon originally located at the 154 bp position. In 10 addition, E. coli codon usage was utilized in this linker. The resulting plasmid obtained from the ligation of these DNA fragments was designated pCMS75.

linker

15 XbaI AvaII  
CTAGATGTCTATCGGTACCGGTGACCGTATCAACACCGTTCTG  
TACAGATAGCCATGCCACTGGCATAGTTGTGGCAAGCACCAG

It should be noted that the DNA sequence of 20 the XbaI-AvaII polylinker includes changes in the DNA sequence for E. coli codon usage. These alternative DNA sequences provide the identical amino acid sequence as the DNA sequence of the Pseudomonas in the same region.

The processed parathion hydrolase is obtained 25 in a soluble form and a molecular weight which corresponded with SDS-PAGE data of approximately 36 Kd.

pCMS77

30 Plasmid pAC1 was digested with ClaI/BamHI and the 4.4kb fragment was isolated from agarose gel. The 1.1 kb ClaI/BamHI fragment that was released by ClaI/BamHI double digest of the pCMS70 plasmid was isolated from agarose gel and subsequently digested with 35 AvaII. This resulted in a 990 bp AvaII/BamHI DNA fragment which was isolated from agarose gel. A

- 20 -

synthetic linker was designed using a ribosomal binding (Shine-Delgarno) DNA sequence that is different from that used in pCMS75. This oligonucleotide, shown below, contains flanking 5' ClaI and 3' AvaII sites.

5

ClaI

CGATTTGATTCTAGAAGGAGGAATAACATATGTCTATCGGTACCGGTGACCGTA-  
TAAACTAAGATCTTCCTCCTTATTGTATACAGATAGCCATGGCCACTGGCAT-

10

AvaII

-TCAACACCGTTCGTG  
-AGTTGTGGCAAGCACCAAG

15 E. coli codon usage was utilized following the methionine codon, ATG, through the AvaII site. The ATG codon was followed by a serine codon originally located at the 154 bp position. This site was identified to be the first amino acid in the N-terminal amino acid sequence of the processed enzyme.

20

Plasmid pCMS77, not shown in Figure 4, is identical to pCMS75, with the exception of the DNA sequence in the regulatory region of the vector pAC1. This change is too small to become apparent in restriction maps such as those in Figure 4.

25

However, such an alteration will enable the formation and aggregation of parathion hydrolase in inclusion bodies rather than in soluble form.

30

E. coli FM5 cells harboring pCMS77 were grown and induced in an identical manner to E. coli FM5 (pCMS75) cells (Example 1). In contrast to E. coli (pCMS75) cells which contained no inclusion bodies the E. coli (pCMS77) cells, had 3-4 large inclusion bodies per cell.

35

SDS-PAGE analysis to determine the proteins found in inclusion bodies was performed. Samples prepared from different fractions of cell lysate were analysed by SDS-PAGE.

- 21 -

Results confirmed the observation that E. coli (pCMS77) yields large amounts of parathion hydrolase which is located in inclusion bodies.

Parathion hydrolase activity of these strains 5 were assayed as described. A ten-fold higher activity was observed using the crude cell extract of E. coli (pCMS75), as compared to the crude cell extract of E. coli (pCMS77).

It is well known that when proteins in cells 10 form an aggregate such as inclusion bodies, the protein may be inactive and require additional processing to obtain proper folding. Therefore, one would anticipate a decrease in specific activity of the crude extract.

15

#### EXAMPLE 1

The present example relates to the effect of metals on parathion hydrolase activity.

Although trace metals may include  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  20 or  $\text{ZnCl}_2$  concentration of 0.015 mM, the addition of larger amounts (i.e. 10-fold increase) of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  and/or  $\text{ZnCl}_2$  result in increased specific activity.

Shaker flask experiments were performed using 25 0.4 liters of BHI medium (Brain Heart Infusion, Difco) containing 100  $\mu\text{g}/\text{ml}$  ampicillin. Flasks were supplemented with 1mM Ca, Cd, Co, Mn, Mg and/or Zn. The cells were grown at 30°C to an  $\text{OD}_{600}$  (optical density at 30 600 nm) =0.4-0.5. Parathion hydrolase activity was induced by raising the culture temperature to 42°C for 1 h and lowering the temperature back to 30°C to 35 continue incubation for another 4 h.

Following growth and induction, cells were centrifuged and resuspended in 20 mM Tris-HCl buffer, pH 8.5, and lysed using a French press (10,000 psi). 35 The supernatant obtained from 30 min centrifugation at 7,000  $\times g$  was used for enzyme assays. (Enzyme assays were performed according the procedure in Serdar and

- 22 -

Gibson, Bio/Technology, 3:570(1985). Additional concentrations of 0.2 mM and 2.0 mM  $\text{CoCl}_2$  were performed. As indicated in Table II, addition of Co and/or Zn greatly increases the specific activity of 5 parathion hydrolase.

TABLE II

	Sample	Specific Activity*
10	Control - no addition of metal	2.0, 2.3, 3.0
	1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.8, 1.9
	1 mM $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$	0.4, 1.0
	1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	1.0, 2.1
15	1 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	3.1, 5.5
	1 mM $\text{ZnCl}_2$	11.5, 19.3
	0.2 mM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	9.8, 15.7
	1.0 mM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	43.2, 46.2, 48.2, 59.0
	2.0 mM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	18.2, 43.2
20		

\*Specific Activity is expressed in  $\mu\text{mol min/mg}$  of protein. The different values correspond to different runs.

Fed-batch fermentation was performed in a 15- liter Biolafitte fermentor. Overnight cultures were inoculated to a final  $\text{OD}_{600}$  of 0.2-0.3 and fermented at 30°C in a 8.0 liter medium consisting of 3.0 mM  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , mineral salts, trace metals, vitamins, yeast extract and glucose as the carbon source. Cells were induced at an 25  $\text{OD}_{600} = 30$  by raising the temperature to 42°C for 1 h followed by 30°C incubation by feeding, pH stabilization (pH 7.0) and agitation until  $\text{OD}_{600}$  reached approximately 60. Cells were harvested, and the parathion hydrolase 30 activity was determined as previously described. The 35 concentration of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  supplemented in this medium is approximately 200-fold greater than normally found in the trace metals solution added to the fermentation medium.

- 23 -

In this experiment, parathion hydrolase activity of 60  $\mu$ mol/min/mg of protein was obtained from an 8.0 liters of cell culture.

5

#### EXAMPLE 2

The present example relates to the purification of parathion hydrolase.

Cultures of P. diminuta (pCMS55) (Serdar, 10 supra, 1985) were grown in TYE-broth (1 percent Bacto-tryptone, 0.5 percent Bacto-Yeast Extract). Antibiotics were used at the following final concentrations ( $\mu$ g/ml); Kanamycin sulfate (Km), 50. The cultures were maintained at 30°C and grown to an  $OD_{600}=8.0$ . The 15 cultures were aerated by shaking at 150 rpm.

The following purification method provides highly purified parathion hydrolase as compared to prior art crude and partial purification methods. A 60-70% 20 purity is demonstrated at the Affi Gel Blue Gel elution step, and a greater than 95% homogeneity is obtained following elution using a sephadex G75 column. The 25 following procedure eliminates the need for detergents such as Tween 20 and Triton X-100.

Cells of P. diminuta (pCMS55) were centrifuged 25 and resuspended in 20 mM Tris-HCl buffer, pH 8.5, (1.0 g cells per 3.0 ml of buffer) and broken by passage through a French pressure cell. The supernatant obtained from 7,000Xg centrifugation for 30 min. was further centrifuged at 100,000Xg for 90 min to give 30 clear crude cell extract.

The cell extract was then applied to the top of a DEAE-Sephadose column. Elution with Tris-HCl buffer gave a solution that contained parathion hydrolase since it does not bind to this column but 35 results in the elimination of many other proteins from the cell extract. The column eluate was applied to the

- 24 -

top of an Affi Gel Blue Gel column. The column was washed with three bed volumes of Tris-HCl buffer. Bound proteins including parathion hydrolase were eluted with linear gradient of NaCl (0.0-0.5 M) in Tris-HCl buffer.

5 Fractions containing parathion hydrolase were pooled and concentrated by ultrafiltration using UM-10 membrane. The concentrated preparation of parathion hydrolase was loaded onto a superfine Sephadex G75 column. Elution was carried out with 20mM Tris-HCl, pH 8.5. Fractions were collected and assayed for 10 parathion hydrolase activity.

Parathion hydrolase can be purified to over 95% homogeneity using this procedure.

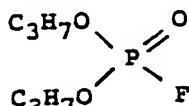
15

### EXAMPLE 3

The present example relates to the hydrolysis of cholinesterase inhibitors by parathion hydrolase.

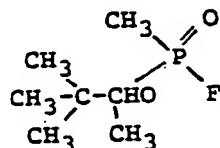
Hydrolysis of cholinesterase inhibitors such 20 as DFP, Sarin and Soman by parathion hydrolase was determined by fluoride ion production. The structures of these cholinesterase inhibitors are as follows:

25



DFP (diisopropyl phosphoro fluoridate)

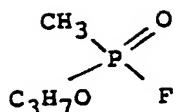
30



SOMAN (pinacolyl methyl phosphonofluoridate)

35

- 25 -



5 SARIN (isopropyl methylphosphono fluoride)

The enzyme preparation used in this study was partially purified parathion hydrolase having a specific activity of 1000  $\mu\text{mol}$  of parathion hydrolyzed per min per mg of 10 protein. The protein concentration of the sample was 10 mg/ml.

METHODS

Two buffers were used. The pH 7 buffer was 15 390 mM in KCl, 40 mM in NaCl, and 20 mM in PIPES. The pH 8.5 buffer was the same in KCl and NaCl, but 20 mM in TRIS. The pH was adjusted with HCl and KOH. The  $\text{F}^-$  sensitive electrode (Orion 96-09) was standardized daily with one or another of these buffers made  $2 \times 10^{-5}\text{M}$  and 20  $2 \times 10^{-4}\text{M}$  in NaF.

The three substrates - DFP, Sarin, and Soman - were prepared as 0.01M solutions in double distilled water, stored at  $-70^\circ\text{C}$ , and brought to  $0^\circ\text{C}$  when ready for use. The enzyme preparation was diluted 200- to 25 4000-fold in appropriate buffer.

A typical determination of enzyme activity using 3mM Soman as the substrate was as follows:

A 10 ml reaction vessel with stirring bar was charged with 3.4 ml pH 7 buffer, and the  $\text{F}^-$  electrode was 30 lowered to within 1 mm of the stirring bar; 1.5 ml 0.01M Soman was added. In about 3 min, the room temperature of 22-24° was attained (determined in trial runs with a thermocouple). Recording was then begun with a stopwatch in cum/split mode. When a low and constant  $\text{F}^-$  release was 35 attained (usually 5-10 min), 100  $\mu\text{l}$  of the 1-200 diluted enzyme sample was added and recording was continued for

another 10-20 min or until not more than 6-8% of the substrate was hydrolyzed. Subtraction of the non-enzymatic slope from the gross slope shows a net enzymatic  $F^-$  change. From this, an enzymatic rate of 5  $\mu$ moles of Soman hydrolyzed per min per ml (or per mg) of undiluted enzyme preparation was calculated.

Where other additions were made (see Table III) small volumes and relatively high concentrations were used so that the added substances 10 could be in contact with the enzyme for 30 min prior to the assay, and then during the assay, at a constant concentration.

The results in the following Tables (III, IV, V) clearly indicate that parathion hydrolase hydrolyzes 15 other organophosphorus compounds such as DFP, Sarin and Soman. The divalent cations tested in this study caused a relatively small (10-20%) inhibition of Soman enzymatic hydrolysis (Table III). Most similar enzymes are stimulated 2- to 20-fold by  $Mn^{2+}$ . EDTA caused 40% 20 inhibition (Table III) at a concentration that would have caused virtually complete inhibition of Soman or DFP hydrolysis by superficially similar enzymes from the squid or from various other microbial and animal sources (Hoskin et al., 1988. Biol. Bull. 175:305). Parathion 25 hydrolase demonstrated the highest activity for Sarin hydrolysis when compared with the activities obtained using purified squid or the obligate thermophile enzyme.

All of the other enzyme sources - mammalian or microbial, partially purified or not - show Soman/DFP 30 hydrolysis ratios of 5-50 (Hoskin et al., 1984. Fundam. Appl. Toxicol. 4(2):165-172), and one, an obligate thermophile does not hydrolyze DFP at all (Chettur et al., 1988. Fundam. Appl. Toxicol. 11:373-380). This 35 ratio of Soman/DFP hydrolysis can be as high as 900-fold (Tables III and V) for parathion hydrolase indicating it has unique properties compared to the previously studied enzymes having activities.

- 27 -

TABLE III  
Hydrolysis of Soman by Parathion Hydrolase

[Soman], mM	pH	Additions	Hydrolysis		μmol/min/mg
			μmoles min <sup>-1</sup> ml <sup>-1</sup>	μmoles min <sup>-1</sup> ml <sup>-1</sup>	
5	5	none	182		18.2
	3	none	94,129		9.4, 12.9
	3	8.5	152		15.2
	3	5 × 10 <sup>-4</sup> M Co <sup>2+</sup>	82		8.2
	3	5 × 10 <sup>-4</sup> M Mn <sup>2+</sup>	90		9.0
	3	5 × 10 <sup>-4</sup> M Zn <sup>2+</sup>	98		9.8
10	3	5 × 10 <sup>-4</sup> M EDTA	67		6.7
	3	none	92		9.2
	2	8.5	101		10.1
	2	none	19.5, 27		2.0, 2.7
	0.5	7	17		1.7
15	0.5	5 × 10 <sup>-4</sup> M Co <sup>2+</sup>			

TABLE IV  
Hydrolysis of Sarin by Parathion Hydrolase

[Sarin], mM	pH	Hydrolysis		μmol/min/mg
		μmoles min <sup>-1</sup> ml <sup>-1</sup>	μmoles min <sup>-1</sup> ml <sup>-1</sup>	
20	4	1140		114.0
	3	1850		185.0
	3	1600		160.0
	25	1310		131.0
	1.6	580		58.0
	1.6	540		54.0
	0.5	580		58.0
	0.4			

TABLE V  
Hydrolysis of DFP by Parathion Hydrolase

[DFP], mM	pH	Hydrolysis		μmol/min/mg
		μmoles min <sup>-1</sup> ml <sup>-1</sup>	μmoles min <sup>-1</sup> ml <sup>-1</sup>	
35	3	4,700	4,867	470.0 486.7
	3	139,000	128,000	13,900.0 12,800.0
	0.5	3,920	4,250	392.0 425.0
	0.5	67,000	67,000	6,700.0 6,700.0

- 28 -

WHAT IS CLAIMED IS:

1. A polydeoxyribonucleotide segment which  
codes for processed parathion hydrolase as shown in

5 Figure 6.

2. A recombinant plasmid adapted for  
transformation of a microbial host said plasmid comprising  
a plasmid vector into which said polydeoxyribonucleotide  
10 segment of Claim 1 has been inserted.

3. The recombinant plasmid of Claim 2 wherein  
the source of the plasmid vector is a microorganism  
selected from the group consisting of the genus  
15 Escherichia, Saccharomyces, Bacillus, Streptomyces and  
Pseudomonas.

4. The recombinant plasmid of Claim 3 wherein  
the microorganism is selected from the group consisting of  
20 the species Escherichia coli, Saccharomyces cerevisiae,  
Bacillus subtilis, Pseudomonas diminuta, and Pseudomonas  
putida.

5. The recombinant plasmid of Claim 4, said  
vector is selected from the group comprising E. coli  
25 plasmid pCMS75 and E. coli plasmid pCMS77.

6. The recombinant plasmid pCMS75 of Claim 5  
as shown in Figure 4.

30 7. A transformed microorganism which includes  
a recombinant plasmid said plasmid comprising a plasmid  
vector into which the polydeoxyribonucleotide segment of  
Claim 1 has been inserted.

35

- 29 -

8. The transformed microorganism of Claim 7  
wherein the microorganism is selected from the group  
consisting of the genus Escherichia, Saccharomyces,  
Bacillus, Streptomyces and Pseudomonas.

5

9. The transformed microorganism of Claim 8  
wherein the microorganism is selected from the group  
consisting of the species Escherichia coli, Saccharomyces  
cerevisiae, Bacillus subtilis, Pseudomonas putida and  
10 Pseudomonas diminuta.

10. The transformed microorganism of Claim 9  
wherein the microorganism is E. coli.

15

11. The transformed microorganism of Claim 10  
wherein said plasmid vector is selected from the group  
comprising E. coli plasmid pCMS75 and E. coli  
plasmid pCMS77.

20

12. Processed parathion hydrolase which is the  
product of the expression in a prokaryotic or eukaryotic  
host cell of an exogenous polydeoxyribonucleotide segment.

25

13. Processed parathion hydrolase as in  
Claim 12 having a homogeneity of greater than 60%.

14. Processed parathion hydrolase as in  
Claim 12 having a homogeneity of greater than 95%.

30

15. A process of purifying soluble parathion  
hydrolase in the absence of detergents.

35

16. The process of Claim 15 wherein the  
detergents are selected from the group consisting of  
Triton X-100 and Tween 20.

- 30 -

17. A process for enhancing the specific activity of parathion hydrolase by the addition to the culture media of greater than .15 mM of a metal selected from the group consisting of cobalt and zinc, or mixtures thereof.

18. The process of Claim 17 wherein the concentration of said metal is in the range of 0.2 mM to 2.0 mM.

10 19. The process of Claim 18 wherein the concentration of said metal is 1 mM.

15 20. The process of Claim 17 wherein the increase in specific activity of crude cell extract is at least 20-fold.

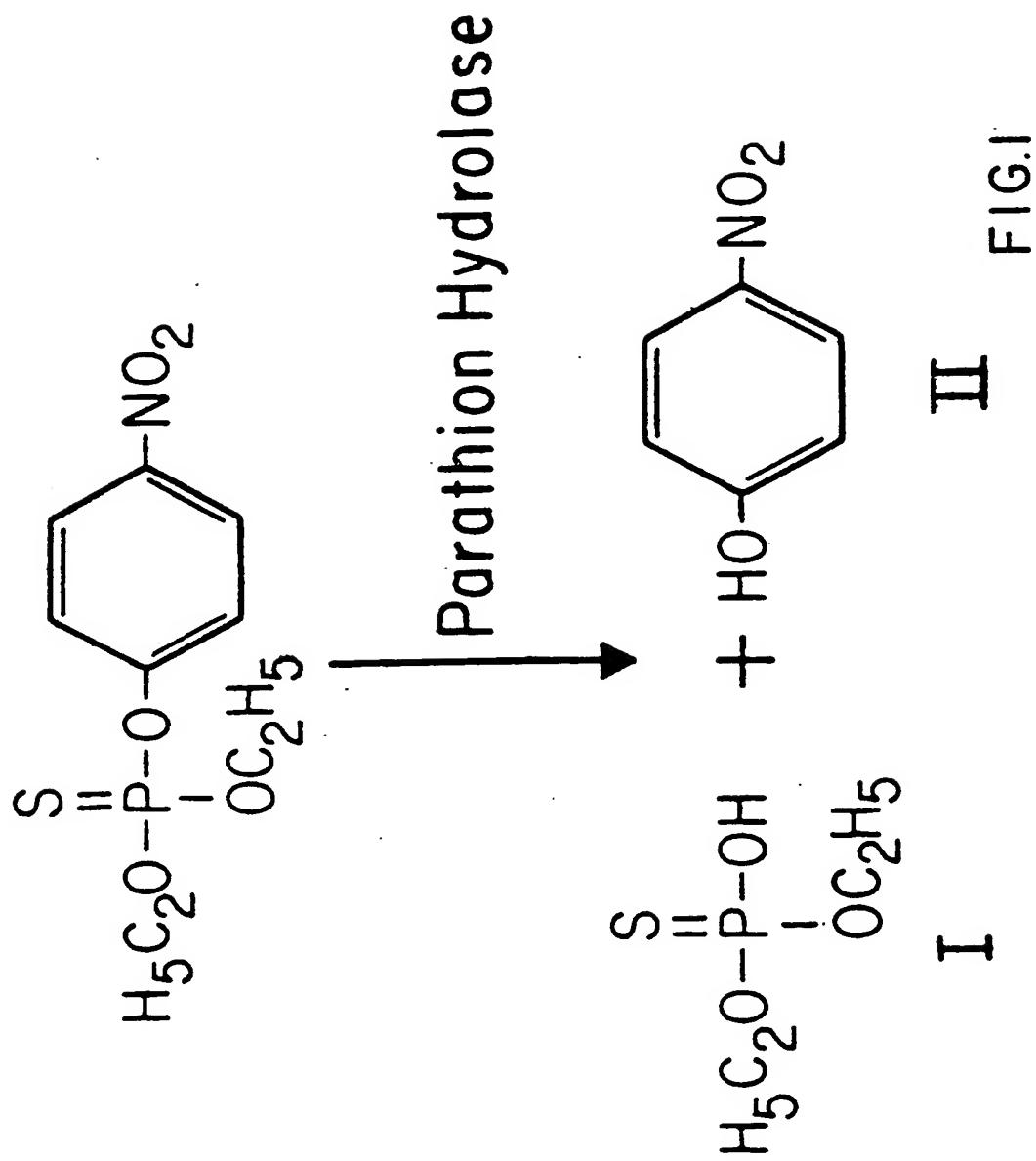
20 21. A polydeoxyribonucleotide segment as in Claim 1 which codes for processed parathion hydrolase as shown in Figure 6 which starts at position 67 base pairs.

25 22. A polydeoxyribonucleotide segment as in Claim 1 which codes for processed parathion hydrolase as shown in Figure 6 which starts at position 154 base pairs.

25 23. A process for the hydrolysis of a cholinesterase inhibitor comprising treating the cholinesterase inhibitor with parathion hydrolase.

30 24. A process as in Claim 23 wherein the cholinesterase inhibitor is one or more of the group consisting of DFP, Sarin and Soman.

1 / 8



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2 / 8

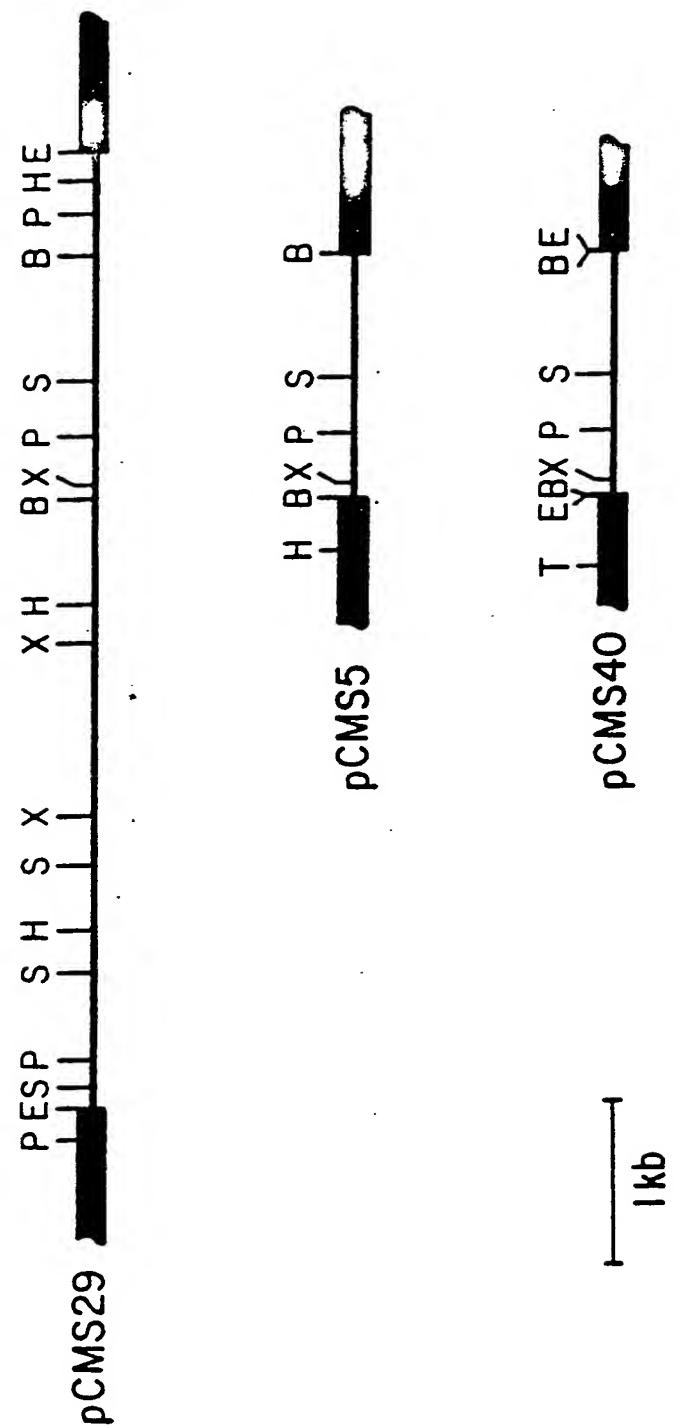
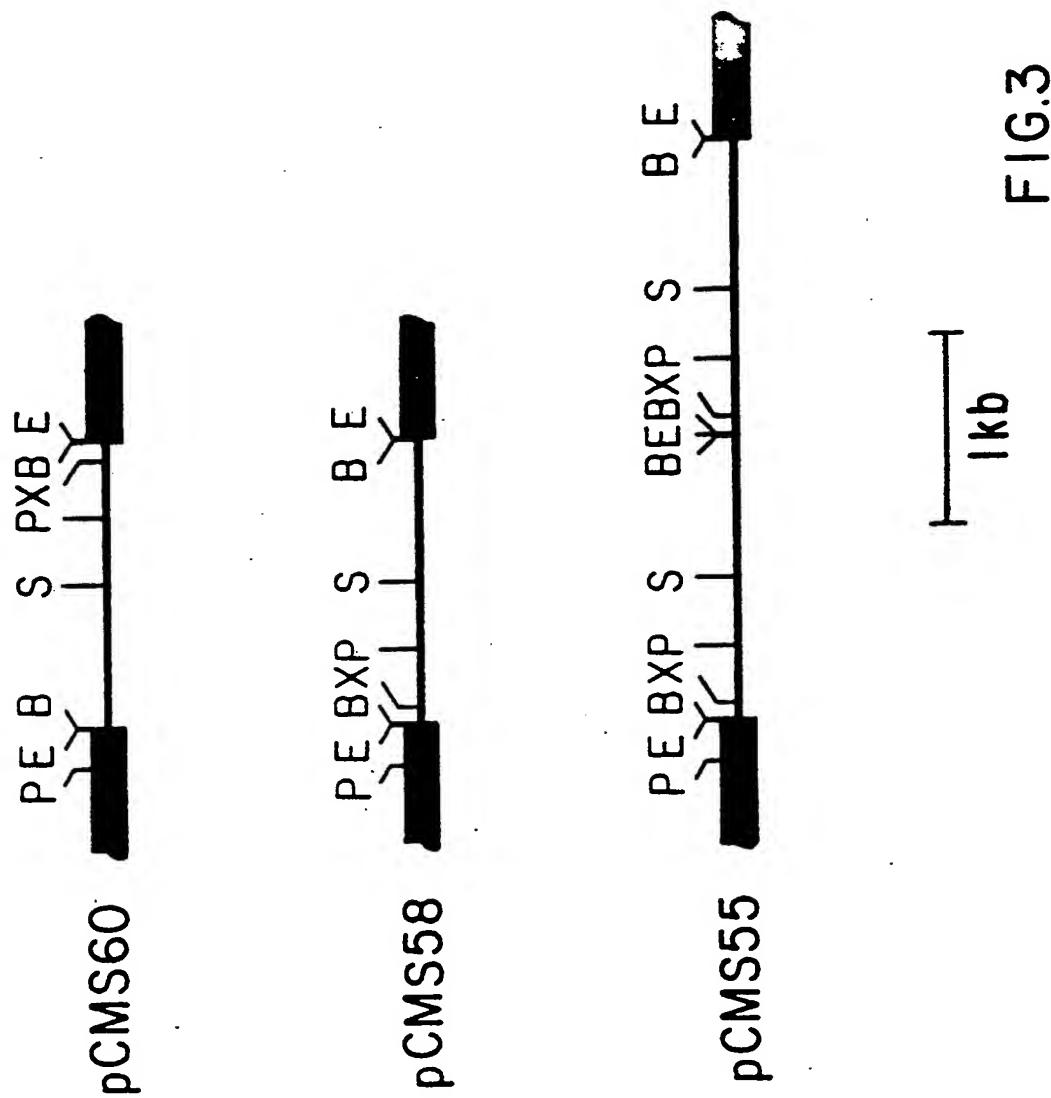


FIG.2

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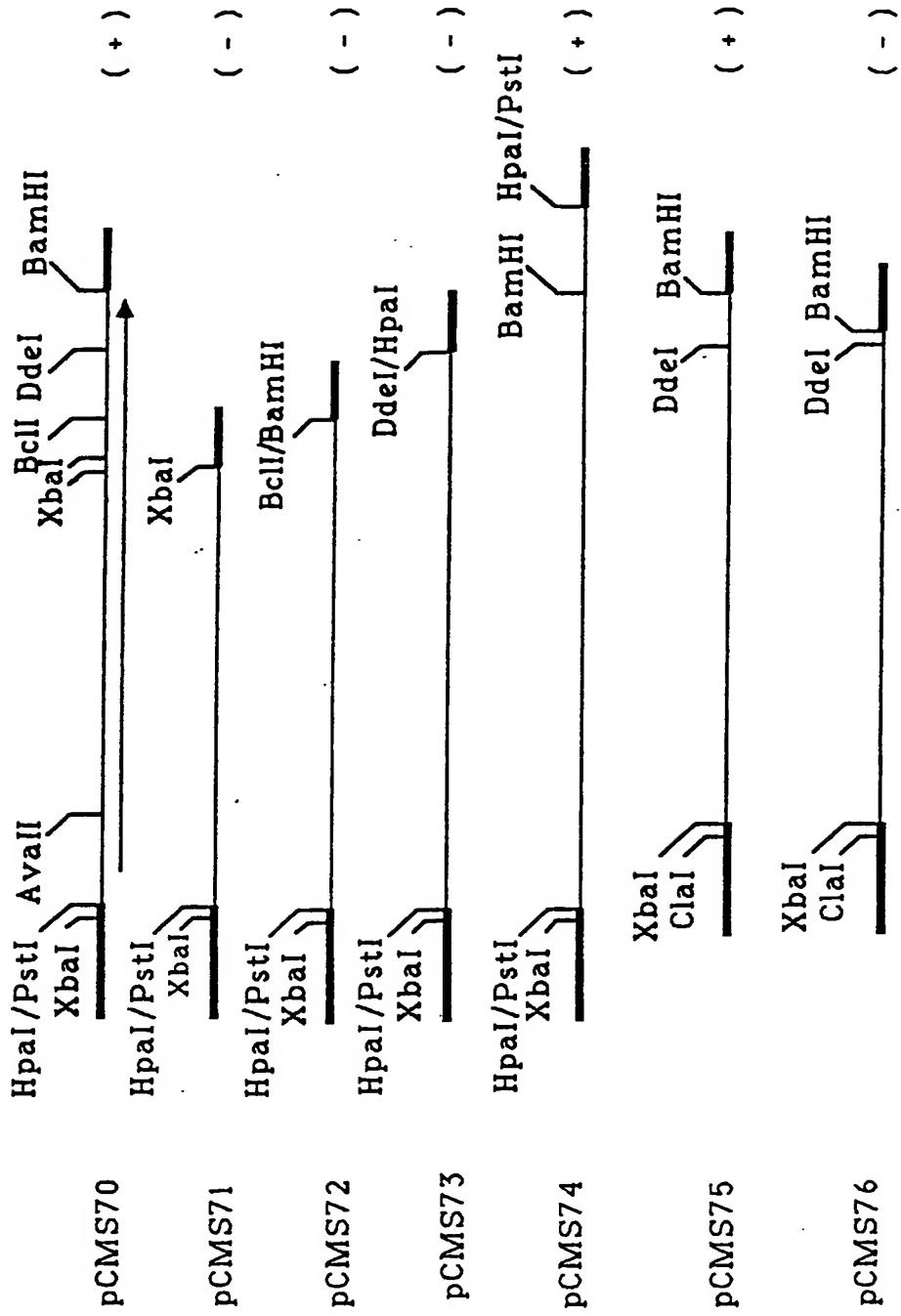
3 / 8



SUBSTITUTE SHEET

4 / 8

## PHENOTYPE



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FIG. 4

5 / 8

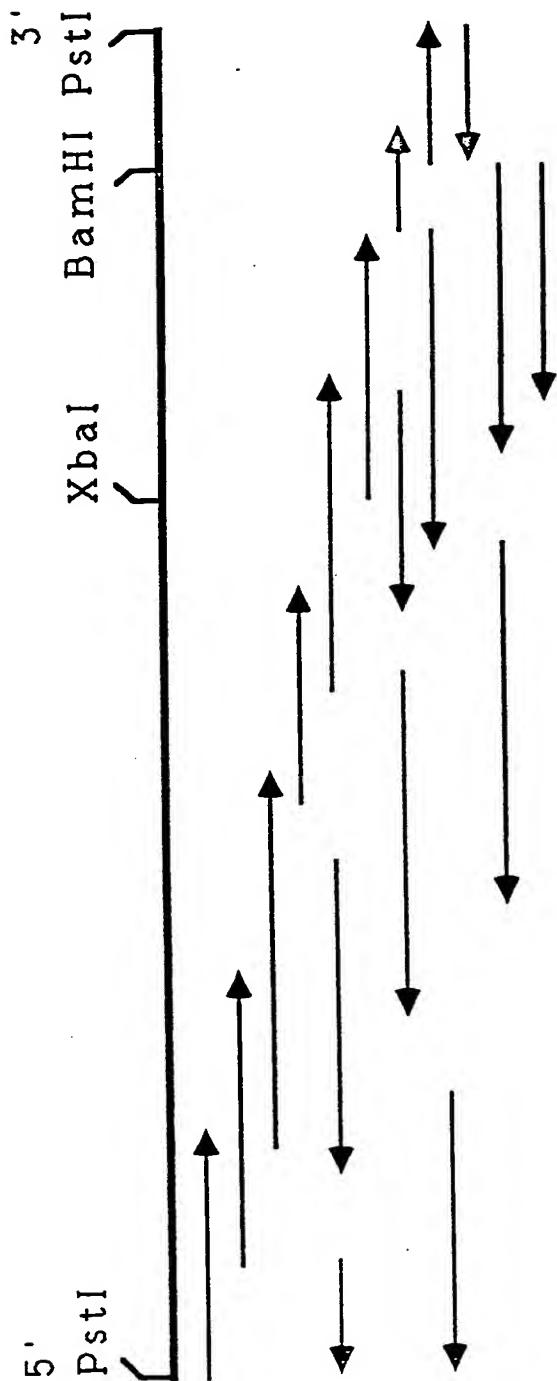


FIG.5

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FIG. 6A

## **SUBSTITUTE SHEET**

7 / 8

FIG. 6B

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8 / 8

FIG. 6C

## **SUBSTITUTE SHEET**

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03721

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): C12N 15/00 U.S. Cl. 435/172.3		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S.	435/172.3, 196, 874 935/14, 29,56	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
Chemical Abstracts, Biological Abstracts, PC/GENE sequence search		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	Eur. J. Appl. Microbiol. Biotechnol. Vol. 8, Nos. 1-2, pp. 103-12 (1979), Munnecke et al., "Production of Parathion Hydrolase Activity."	1-24
X	US, A 4,391,887, BAUMGARTEN ET AL., 05 July 1983 (05.07.83).	23,24
X	Bio/Technology, Vol. 3, No.6, pp. 567-71 (1985), Serdar et al., "Enzymatic Hydrolysis of Organophosphates: Cloning and Expression of a Parathion Hydrolase Gene from Pseudomonas diminuta".	1-4,7-10,21 <u>22</u> 5,6,11-14
* Special categories of cited documents: <sup>14</sup>		
"A" document defining the general state of the art which is not considered to be of particular relevance		"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document but published on or after the international filing date		"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means		"A" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
07 December 1989	19 JAN 1990	
International Searching Authority	Signature of Authorized Officer	
ISA/US	Robert A. Wax 	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Appl. Environ. Microbiol., Vol. 51, No.5, pp. 926-930 (1986), Mulbry et al., "Identification of a Plasmid-Borne Parathion Hydrolase Gene from <i>Flavobacterium</i> sp. by Southern Hybridization with opd from <i>Pseudomonas diminuta</i> ."	1-24
X	Appl. Environ. Microbiol., Vol. 53, No.7, pp. 1685-1689 (1987), Attaway et al., "Bacterial Detoxification of Diisopropyl Fluorophosphate".	23,24
A	Plasmid, Vol. 18, No. 2, pp. 173-177 (1987) Mulbry et al., "Physical Comparison of Parathion Hydrolase Plasmids from <i>Pseudomonas diminuta</i> and <i>Flavobacterium</i> sp."	1-24
X	J. Bacteriol., Vol. 170, No.5, pp. 2306-2311, (May 1988), McDaniel et al., "Cloning and Sequencing of a Plasmid-Borne Gene (opd) Encoding a Phosphotriesterase."	1-4,7-10, 12-14,21,22 5,6,11,15,16 23,24
P, A	Appl. Environ. Microbiol., Vol. 54, pp. 2586-2589(1988), Harper et al., "Dissimilar Plasmids Isolated from <i>Pseudomonas diminuta</i> MG and a <i>Flavobacterium</i> sp. (ATCC 27551) Contain Identical opd Genes."	1-24